The influence of pH and selected cations on the spectrofluorometric determination of oxytetracycline hydrochloride

(INDEX: 104-110/2010 Copernican Letters®Vol 1)

Anna Piasta, Anna Filipiak-Szok, Marzanna Kurzawa, Edward Szłyk
Wydział Chemii, Uniwersytet Mikołaja Kopernika
Ul. Gagarina 7, 87-100 Toruń,
e-mail: aniapiasta@wp.pl

Abstract
The spectrofluorometric method for the oxytetracycline hydrochloride in pure and in veterinary products Tetrox and Oxymed 50 determination is described. The influence of pH solution and presence selected cations on fluorescence intensity were studied too. It was ascertained that the highest fluorescence intensity take place at pH=9. Moreover, the quenching of fluorescence intensity was observed at presence Ca^{2+}, Al^{3+} and Fe^{3+} in contrast to Mg^{2+} which caused increasing of intensity. The obtained recovery (97.23±0.12% for Tetrox and 95.21±0.10% for Oxymed 50) values meet the European Pharmacopoeia requirement. Moreover, the relative standard deviation (RSD) was below 0.23% confirmed high precise of the method. The statistical test (t-Student and F-Snedecor) used for comparison spectrofluorometric and chromatographic methods pointed that they are comparable in respect of precision but not of accuracy.

Keywords: *oxytetracycline hydrochloride, fluorometry*

1. Introduction

Tetracyclines (TCs), among these oxytetracycline hydrochloride (OTC), are used routinely in veterinary medicine for prevention and control of disease and have a good activity against acute disease caused by Gram-positive and Gram-negative bacteria. Various analytical methods have been reported for the determination of these compounds. European Pharmacopoeia recommends liquid chromatography for determination of OTC in pure and dosage forms [1-4].

In literature, the main group of methods applied for tetracycline determination are chromatographic method [5-11]. Chromatography was used for analysis residues of TCs in human and animal tissues as well for purity and assay of above antibiotic in pure and pharmaceutical forms [6-9]. Moreover, the spectroscopic procedure were described [l2- 18]. Among these there are: spectrophotometric [15-18] and fluorometric [12-14]. The fluorometric methods were based on natural fluorescence properties of above antibiotics or on the formation of complexes tetracyclines with several metals, which revealed fluorescence [19-23]. All fluorometric method characterized the high sensitive and precise. Authors of cited reports pay attention on the influence many parameters on the fluorescence intensity, linearity and sensitivity.

The main goal of this paper was definition of the influence pH and selected cations on the oxytetracycline hydrochloride determination in pure form and in formulations. The obtained results were compared with these obtained by pharmacopoeial (HPLC) one and discussed with regards of accuracy and precision.

2. Experimental

2.1. Chemicals

The oxytetracycline hydrochloride was purchased from SIGMA ALDRICH (Germany) whereas veterinary products (Tetrox and Oxymed) from local veterinary pharmacy. All used reagent and solvent (analytical grade) were from POCH (Gliwice, Poland).

2.2. Apparatus

Fluorometric measurements were performed on a Hitachi F-7000 Fluorescence Spectrophotometer using a Xenon lamp source for excitation in the 1 cm quartz cell. Excitation and emission slit widths were 5 nm for the wavelength response of the system. Individual and three-dimensional spectra were recorded in the range 200–900 nm. The spectra were evaluated with the PC software package supplied with spectrophotometer (FL-Solution 2.1 for F-7000).

HPLC apparatus consisting of Shimadzu LC-10A system equipped with a model LC-10AT pump, an SPD-M20A variable wavelength detector and the auto sampler SIL-20AC HT was used for development and evaluation of this method. The chromatographic data were recorded and processed by the *LCsolution version 1.23 SP*. Chromatographic separation was performed using a Supelco C-18 column (150mmx 4.6mm i.d., 5 µm particle size) with isocratic elution. The mobile phase consisted of a mixture of: 90 mL 2-methyl-2-propanol; 60 mL phosphate buffer (pH=7.5): 50 mL tetrabuthyloammonium hydrosulphate: EDTA diluted up to 1L with bidistilled water. The flow rate was 1.3 ml min^{-1} with detection at 254 nm. The HPLC system was operated at room temperature (25°C). Peak identity was confirmed by retention time comparison.

2.3. Influence of oxytetracycline hydrochloride concentration on the fluorescence intensity

The measurements in the concentration range from 2 to 25 mg/L of OTC were made for the definition of the concentration influence on fluorescence intensity. The excitation wavelength was 255 nm and emission 450 nm.

2.4. Influence of pH on the fluorescence intensity

For the purpose of definition the influence of pH on the fluorescence intensity of oxytetracycline hydrochloride ten buffer solutions at pH from 2 to 11 were prepared. The OTC solution was prepared by dissolving 50.0 mg of antibiotic in water in 1L volumetric flask resulting $1.0 \cdot 10^{-4}$ M. Next, 2 mL of the solution was placed into 10 mL volumetric flask and made up to volume with particular buffer solution. The emission spectra were recorded after sample excitation at $\lambda_{ex.}$ = 255 nm.

2.5. Influence of cations on the fluorescence intensity

It is known that tetracyclines (among these oxytetracycline) create the complexes with di- and trivalent metals [19]. Because of in veterinary use tetracycline are dosed orally as water solution, we would like to study the effect of selected cations (existed in water) such as: calcium, magnesium, aluminium and iron on the fluorescence of oxytetracycline.

For this purpose, the $1.0 \cdot 10^{-4}$ M solutions of above cations were prepared by dissolving of adequate amount of theirs salt. Next, 2 mL of OTC ($1.0 \cdot 10^{-4}$ M) and 2 mL of cation solution were placed in 10 mL volumetric flask and made up to volume by bidistilled water. The spectra of above solutions were recorded after 10 min.

2.6. Sample preparation

Tetrox and Oxymed 50 for the spectrofluorometric determination were prepared by dissolving in buffer solution (pH=9) appropriate amount of veterinary preparations for the concentration of active substance were $1 \cdot 10^{-4}$ M. This solution was diluted with buffer in 10 mL volumetric flask giving following concentration 5.0; 10 and 15 mg/L.
In the case of chromatographic analysis, the working solutions were 40, 0; 80,0 and 120 mg/L. They were prepared by diluting of stock solution ($1 \cdot 10^{-4}$ M) with water in 10 mL volumetric flask.

2.7. Chromatographic analysis

High performance liquid chromatography was applied as references method (pharmacopoeial). The analysis was carried out by calibration curve method. For this purpose, seven solutions at the concentration range from 20 to 200 mg/L were prepared. The calibration curve was made as the dependence the peaks area on the concentration of OTC. The equation of calibration curve was y = (12558±100)x + (501746±13276) with linear regression values r^2 = 0.9998 and LOD and LOQ 3.36 and 10.08 mg/L, respectively. Limit of detection (LOD) was calculated from $(y + 3 \cdot s_{y/x})/b$, where the calculated intercept of the calibration line can be used as an estimate of y; $s_{y/x}$ being the standard deviation in the *y*-direction of the calibration line and b being the slope of the calibration curve. The $10 \cdot s_{y/x}/b$ expression was used for estimation of the quantification limit, (LOQ) [25-26].

3. Results and discussion

3.1. Spectrofluorometry

3.1.1. Influence of OTC concentration

The dependence of fluorescence intensity on the OTC concentration is presented at Fig.1.

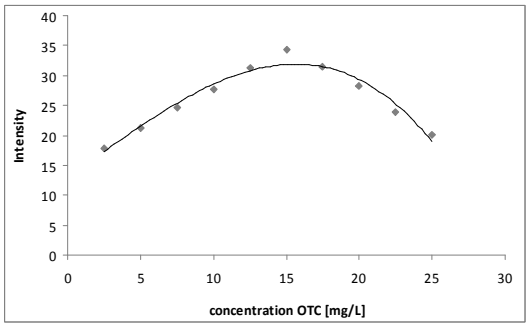

Fig.1. The correlation between fluorescence intensity and OTC concentration

As it is seen at the Fig.1. linear correlation between fluorescence intensity and OTC concentration was required in the range from 2 to 15 mg/L. For more concentrated solution the quenching of fluorescence take place. For this reason the calibration curve was plotted only in this range. The equation of calibration curve was found as follows: y = (2.2581±0.0260)x +(7.5753±0,2529) with determination coefficient r^2 = 0.9995. The calculated limit of detection and determination were 0.40 and 1.20 mg/L, respectively.

3.1.2. Influence of pH

The obtained spectra are presented at the Fig.2.

On the basis of obtained spectra it was observed that their shapes and intensity were different. For solutions at pH= 2 and pH=3 maximum of λ_{em} exist at 496 nm. In the other cases (excluding pH= 6 and pH=7) the second maximum appeared at 455 nm. It was ascertained that the biggest fluorescence intensity was at pH=9.

3.1.3. Influence of cations

At the Fig. 3 the spectra for oxytetracycline solution and for OTC with individual cations are presented. The excitation wavelengths for prepared solutions were different than for pure OTC what confirmed that complexes between oxytetracycline and studied cations were created. It was observed that maxima at emission spectra were shifted too.

Additionally, at the Fig. 4. the changes of fluorescence intensity of OTC under of studied cations influence are presented. The concentration of OTC and studied cations were the same and amounted $2 \cdot 10^{-5}$M. On the basis of obtained results it was verified that Ca^{2+}, $Al^{3+}$and Fe^{3+} cations caused fluorescence quenching in contrast to Mg^{2+}.

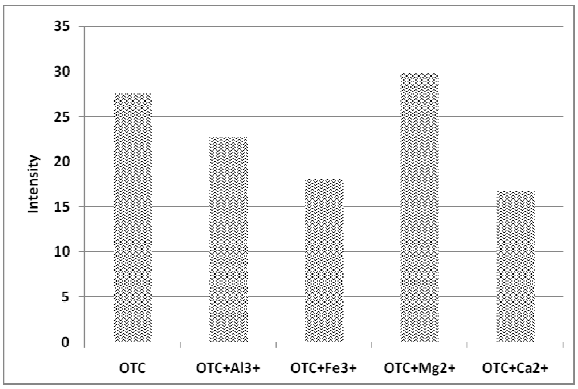

Fig.4. The changes of OTC fluoroscence intensity in the presence of cations ($\lambda_{em.}$= 455 nm)

3.1.4. Analysis of veterinary preparations

Two veterinary products (Tetrox and Oxymed 50) were analyzed to the check of spectrofluorometric method. The measurements were carried out fivefold for each solution. The obtained results are presented in Tab.1.

The average recoveries obtained for this determination were as follows: 97.23% and 95.21% for Tetrox and Oxymed 50, respectively and the calculated RSD values were less than 0.5%. These results testify good accuracy and high precision of tested method and can be accepted for pharmaceutical analysis of oxytetracycline hydrochloride in the studied preparations. Moreover, the recovery values are in the range recommended by British Pharmacopoeia (BP) (95-100.5%) and one can conclude that this method could be suitable for determination this antibiotic in the studied veterinary products.

3.2. HPLC

For the check of spectrofluorometric method and to verify obtained results, HPLC method as reference (pharmacopoeial) one was applied. For this reason the same veterinary preparation were analyzed. Three solutions at the concentration 40, 80 and 120 mg/L of Tetrox and Oxymed 50 were prepared. Each solution was analyzed fivefold and results are presented in Tab. 2.

The obtained average recovery values were 99.36% for Tetrox and 99.32 % for Oxymed 50 and are in accordance recommended by BP. Similar situation is observed for precision of elaborated method. The average values of relative standard deviation calculated for studied pharmaceutical preparations were 0.08% for Tetrox and 0.43% for Oxymed 50 and are acceptable by BP (RSD < 5.0%) for determination of OTC in veterinary preparations.

3.3. Comparision of spectrofluorometric and chromatographic methods

The spectrofluorometric method was compared with pharmacopoeial in respect of precision and accuracy by t-Student and f- Snedecor tests [26].

Comparing both method by statistical test, it should be noted that the they are comparable in respect of precision ($F_{calc.}$< $F_{tab.}$). Unfortunately, the t-Student test indicated that the accuracy (expressed as recovery) of spectrofluorometric analysis is significant different then chromatographic method.

4. Conclusions

Concluding, the great advantages of proposed methods are small amount of sample needed for total analysis and short time of measurement. The data obtained from the described procedures prove good accuracy and reasonable precision (acceptable by BP). The spectrofluorometric method for determination of oxytetracycline hydrochloride in veterinary product Tetrox and Oxymed 50 method can be comparable with chromatographic one only in respect of precise but not in accuracy. Moreover, the obtained results pointed on significant influence the pH of analyzed solution and the presence cations on fluorescence intensity.

Table 1.

The results of oxytetracycline hydrochloride determination in Tetrox and Oxymed 50

Theoretical [mg/dm^3]	Determined * [mg/dm^3]	Recovery** [%]	RSD** [%]
	Tetrox		
5.00	4.87±0.06		
10.00	9.74±0.09	97.23±0.12	0.23
15.00	14.59±0.12		
	Oxymed 50		
5.00	4.76±0.06		
10.00	9.52±0.12	95.21±0.10	0.18
15.00	14.27±0.18		

*n= 5; **n=15

Table 2.

The results of oxytetracycline hydrochloride determination in Tetrox and Oxymed 50

Theoretical [mg/dm^3]	Determined * [mg/dm^3]	Recovery** [%]	RSD** [%]
	Tetrox		
40.00	39.73±0.43		
80.00	79.55±0.58	99.36±0.04	0.08
120.00	119.15±0.65		
	Oxymed 50		
40.00	38.46±0.47		
80.00	76.81±0.55	96.32±0.23	0.43
120.00	116.15±0.62		

*n= 5; **n=15

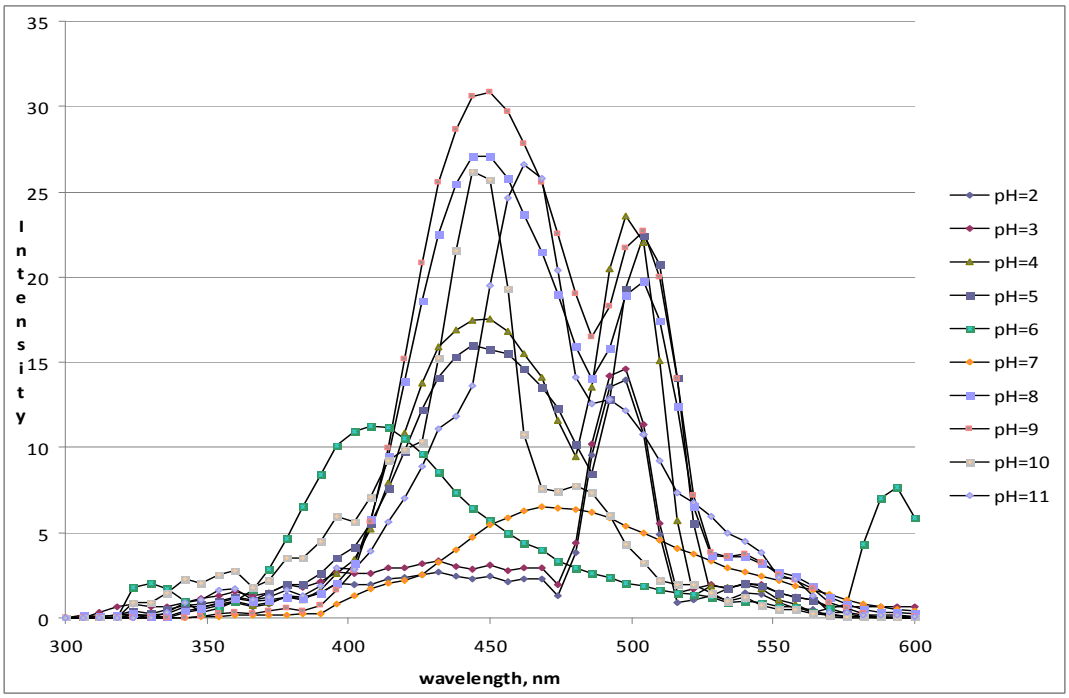

Fig. 2. The fluorescence spectra of OTC at various pH values (c_{OTC} =2·10^{-5})

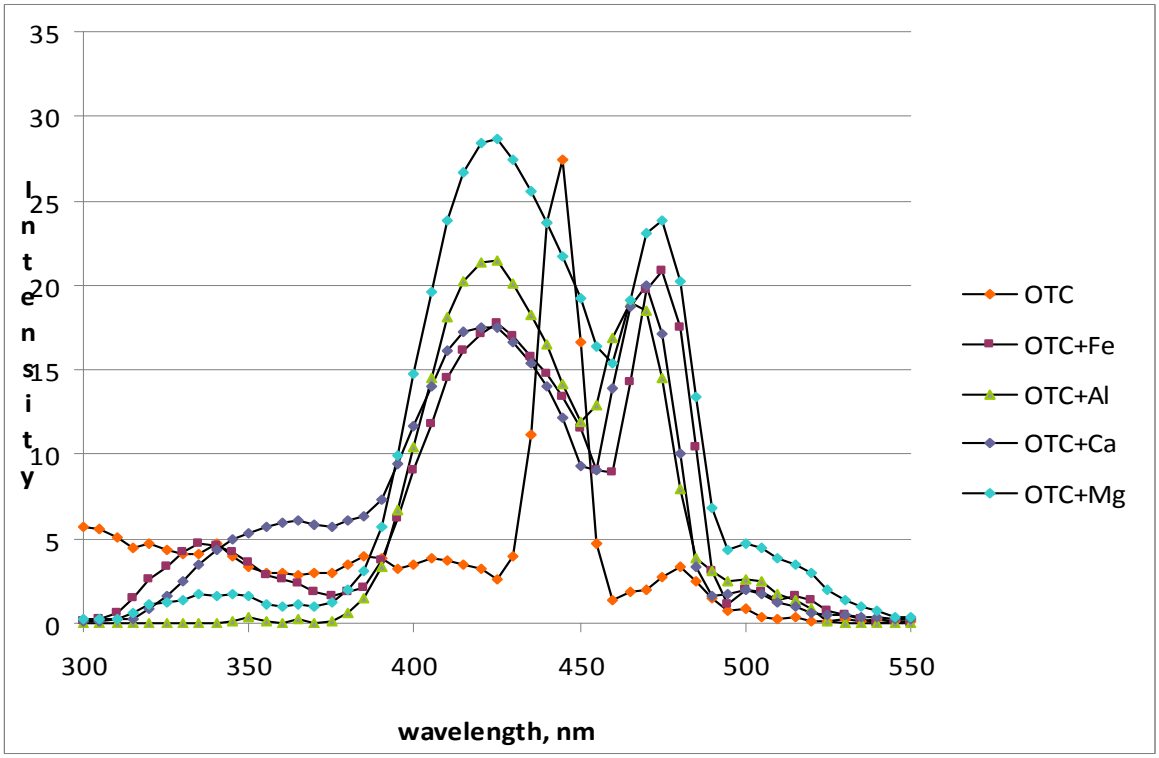

Fig. 3. The emission spectra ($\lambda_{em.}$= 455 nm) of OTC (2·10^{-5}M) with Ca^{2+}, Mg^{2+}, Al^{3+}, Fe^{3+} (2·10^{-5}M)

5. References

[1]A.L. Cinquina, F. Longo, G. Anastasi, L. Giannetti, R. Cozzani , *Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle,* Journal of Chromatography A, 987 (2003) 227–233

[2] H.C. Goicoechea, A.C. Olivieri, *Selection of the wavelength range and spectrophotometric determination of leucovorin and methotrexate in human serum by a net analyte signal based method,*Anal. Chem. 71 (1999) 4361.

[3] I. Garc´ıa, L. Sarabia, M. Cruz Ortiz, Anal. Chim. *Detection capability of tetracyclines analysed by a fluorescence technique: comparison between bilinear and trilinear partial least squares models* , Acta 501 (2004) 193.

[4] European Pharmacopoeia, Cansil of Europe, Strasbourg 2005 I 2006; Vol. 1, 1270-1271; 1482-1485; *Suplement 5*, 4286-4288, 4310-4311.

[5] S.B. Levy, *Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle* ,J. Food Protect. 50 (1987) 616.

[6] R.D. Caballero,J.R. Torres-Lapasió, M.C. García-Alvarez-Coque , G. Ramis-Ramos , *rapid liquid chromatographic determination of tetracyclines in animal feeds using a surfactant solution as mobile phase* , Analytical Letters, 35(4), 687-705 (2002).

[7] M. Touraki , P. Rigas ,P. Pergandas ,C. Kastritsis , *Determination of oxytetracycline in the live fish feed Artemia using high-performance liquid chromatography with ultraviolet detection* , Journal of Chromatography B, 663 (1995) 167-171.

[8]L.I. Sokolova , A.P. Chernyaev, *Determination of Benzylpenicillin, Levomycetin (Chloramphenicol), and Tetracycline in Food Products by High-Performance Liquid Chromatography*, Journal of Analytical Chemistry, Vol.56, No.11, 2001, pp.1032-1034.

[9] H.T. Lu, Y. Jiang ,H.B. Li, Chen F., Wong M.H., *Determination of Multi-Residues of Tetracyclines and Their Metabolites in Milk by High Performance Liquid Chromatography-Tandem Positive-ion Electrospray Ionization Mass Spectrometry* , Chromatographia 2004, 60, 259-264.

[10] J. E. Sokol, J. Matisova, *Determination of tetracycline antibiotics in animal tissues of food-producing animals by high-performance liquid chromatography using solid-phase extraction*, Chromatogr. A 669 (1994) 75.

[11] W.H. Farrington, J. Tarbin, J. Bygrave, G. Shearer, *Analysis of trace residues of tetracyclines in animal tissues and fluids using metal chelate affinity chromatography/HPLC*, Food Addit. Contam. 8 (1991) 55.

[12] A. Pena, A. Carmona, A. Barbosa, C. Lino, I. Silveira, B. Castillo, *Determination of tetracycline and its major degradation products by liquid chromatography with fluorescence detection*, J. Pharm. Biomed. Anal. 18 (1998) 839–845.

[13] W. Croubles, C.V. Petheghem, W. Baeyens, *A stability study of tetracycline and tetracycline cyclodextrins in tablets using a new HPLC method,* Analyst 119 (1994) 2713–2716.

[14] R.J. McCracken, W.J. Blanchflower, S.A. Haggan, D.G. Kennedy, *Determination of tetracycline and its major degradation products by liquid chromatography with fluorescence detection*, Analyst 120 (1995) 1763–1766.

[15] J.O. De Beer, J. Hoogmartens, *Tetracycline, oxytetracycline and chlortetracycline determination by flow injection potentiometry,* J. Pharm. Biomed. Anal.11 (1993) 1239–1250.

[16] P.D. Bryan, J.T. Stewart, *Validation of a simple method for the determination of oxytetracycline in ointment by non-aqueous capillary electrophoresis* , J. Pharm. Biomed. Anal. 12(1994) 675–692.

[17] S.A. Al-Tamrah, A.A. Alwarthan, *Tetracycline, oxytetracycline and chlortetracycline determination by flow injection potentiometry,* Anal. Lett. 25 (1992)1865–1876.

[18] R. Karlicek, P. Solich, *Flow-injection spectrophotometric determination of tetracycline antibiotics,* Anal. Chim. Acta 285 (1994) 9–12.

[19] W. Hinrichs, C. Kisker ,M. Du¨vel, A. Mu¨ller , K. Tovar ,W. Hillen , W. Saenger , *Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor*, Science 264:418–420

[20]M.Takahashi , L. Altschmied ,W. Hillen , *Noninducible Tet Repressor Mutations Map from the Operator Binding Motif to the C Terminus*, J Mol Biol 187:341–348

[21] C. Kisker , W. Hinrichs , K. Tovar , W. Hillen , W. Saenger , *Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor*, J Mol Biol 247:260–280

[22] P. Orth , F. Cordes ,D. Schnappinger , W. Hillen , W. Saenger , W. Hinrichs , *Two mutations in the tetracycline repressor change the inducer anhydrotetracycline to a corepressor*, J Mol Biol 279:439–447

[23]S.J. Swamy and B.K. Kumar, *Synthesis, spectroscopic characterization and antibacterial activity of lanthanide–tetracycline complexes*, Ind. J. Chem., 34A, 235 (1995).

[24] D.E. Fenton, S.J. Kitchen and C.M. Spencer, *Nuclear Magnetic Resonance Spectroscopy*, Inorg. Chim. Acta, 139, 55 (1987).

[25] Ufnalski W., *Excel dla chemików i nie tylko*, WNT, Warszawa 2002.

[26] Czerminski J.B., *Metody statystyczne dla chemików,* PWN, Warszawa 1986; 414-417.